

LLNA/EC3 Validation

David Basketter¹, Frank Gerberick² and Ian Kimber³

¹St John's Institute of Dermatology, St Thomas' Hospital, London, UK

²Procter & Gamble, Cincinnati, USA

³Syngenta CTL, Alderley Park, Macclesfield, Cheshire, UK

Following the formal validation of the local lymph node assay (LLNA) as a method for hazard identification by ICCVAM and ECVAM (NIH, 1999; Gerberick et al, 2000; Balls and Hellsten, 2000; Dean et al, 2001), and its subsequent enshrinement in regulatory guidelines (OECD, 2002), considerable further evaluation and development of the LLNA has taken place. Most notably, this has been in the use of the LLNA to determine relative potency, so that potential skin sensitizers may be ranked and to provide a key input for skin sensitization risk assessment. As a consequence, it has been proposed to perform a validation of the potency measurements provided by the LLNA. For this purpose the following questions are addressed in this dossier:

Q1: In those circumstances where an evaluation of skin sensitization potency is required for risk assessment purposes, do EC3 values derived from linear interpolation of LLNA dose response data provide an appropriate and reliable approach?

Q2: If yes, do EC3 values provide a suitable method for ranking of contact allergens according to skin sensitization potency?

Q3: If yes, does ranking of potency based on LLNA-derived EC3 values correlate with available human data and clinical experience?

Background

For the prediction of skin sensitisation potential, the local lymph node assay (LLNA) was proven several years ago to be a fully validated alternative to guinea pig tests. More recently, information from LLNA dose response analyses has been used to assess the relative potency of skin sensitising chemicals. These data are then deployed for risk assessment and risk management. EC3 measurements are reproducible in both intra- and inter-laboratory evaluations and are stable over time. It has been demonstrated also, by several independent groups, that EC3 values correlate closely with data on relative human skin sensitisation potency. In this dossier, the validity of these relative potency measurements are reviewed. It is concluded the LLNA conducted following the principles of OECD Guideline 429 does provide a valuable assessment of relative sensitising potency in the form of the EC3 value (estimated concentration of a chemical required to produce a 3-fold stimulation of draining lymph node cell proliferation compared with concurrent controls), and that all reasonable validation requirements have been addressed successfully. Consequently, the recommendation made here is that LLNA EC3 measurements should now be regarded as a validated method for the determination of the relative potency of skin sensitising chemicals.

Introduction

The LLNA has been formally validated and adopted into OECD guidelines. The internationally accepted method presented in Guideline 429 follows the standard protocol published 10 years earlier (Kimber and Basketter, 1992), but allows also for the use of a greater number of mice per group and pooling of nodes from individual animals. It also foresees the use of an alternative (radioactive) endpoint should it prove to be equally sensitive as the ³HTdR employed in the standard assay. All the discussion that follows concerning the possibility of ranking potency in the LLNA draws on knowledge derived from LLNAs conducted according to OECD Guideline 429. In the few instances where this is not the case but it is felt that the information makes an important contribution, it has been clearly indicated with any limitations identified.

It is not appropriate here to review any aspect of the validation of the LLNA for basic “yes/no” hazard identification or to present a detailed protocol since this is now well established (NIH, 1999; Gerberick et al, 2000; Balls and Hellsten, 2000; Dean et al, 2001). However, it is worthwhile recalling why the classification threshold for this binary decision was set at a stimulation index (SI) value of 3. The SI itself simply represents the ratio of ³HTdR counts in the test group compared to those in the concurrent vehicle treated control. In the earliest phase of assay development, it was judged that an SI of 3 was the point where a clear activation signal could be separated from the inherent biological noise. With greater experience and testing of greater numbers of chemicals, it became clearer that this value represented a good point of discrimination between sensitisers and irritants/non-sensitisers. Ultimately, a retrospective analysis of over a hundred chemicals confirmed that an SI of 3 was an appropriate, if slightly conservative, threshold (Basketter et al, 1999). It is worth noting that other workers, using a non-OECD compliant version of the LLNA (³HTdR incorporation is measured *in vitro* in a manner very similar to the earliest published work on the LLNA in the late 1980s) have also found an SI of 3 a suitable threshold for the identification of skin sensitising chemicals (eg van Och et al, 2000; De Jong et al, 2002).

Since the original validation of the LLNA, data on a considerable number of chemicals have been generated. Much of this work has been placed in the public domain via the peer reviewed literature (eg Gerberick et al, 2005; Basketter et al, 2007; Anderson et al, 2007). All of these publications have successfully used an SI value of 3 as a means of identifying skin sensitising chemicals. Currently, a further manuscript is being published which adds approximately 100 further chemicals to the database and provides the corrections to the original Gerberick et al paper published at the end of 2005. The corrections are already available as are many of the new chemicals and so these are presented in Appendix 1. As this new data is still being compiled for publication, it has not been subjected to detailed analysis here.

Beyond these considerations however has emerged the question of whether and to what extent the quantitative output of the LLNA might also be used to provide some indication of the strength of a skin sensitiser. These thoughts were first fully encapsulated in a publication in 1997, where the concentration of the known potent allergen 2,4-dinitrochlorobenzene necessary to generate a LLNA threshold response was contrasted with that of the OECD weak positive control allergen, hexyl cinnamic aldehyde (Kimber and Basketter, 1997). The 160 fold difference in these concentrations was felt to be important and led to much further investigation, the culmination of which is encapsulated in the pages which follow. It is important to mention that this type of analysis is common in many other toxicology endpoints.

Data to support LLNA as a reliable and robust approach for skin sensitization dose response analysis

The protocol for the determination of the LLNA EC3 value is as follows. Essentially, the method represents a simple linear interpolation of the points in the dose response curve that lie immediately above and below the classification threshold, ie a stimulation index of 3. If the data points lying immediately above and below the SI value of 3 have the co-ordinates (a,b) and (c,d) respectively, then the EC3 value may be calculated using the equation: $EC3 = c + [(3-d)/(b-d)](a-c)$. This is represented graphically in Figure 1. Where this equation cannot be applied, then an approach to model a limited degree of extrapolation of LLNA dose response data can be deployed (Ryan et al, 2007).

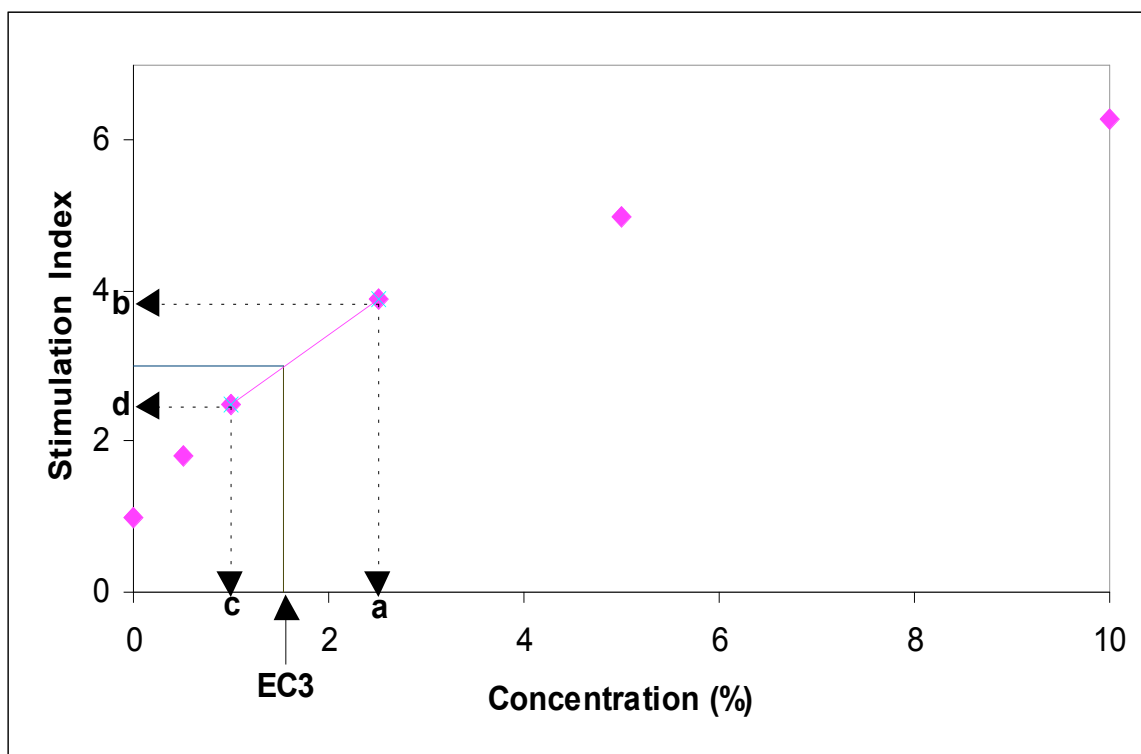


Figure 1. The calculation of the LLNA EC3 value by linear interpolation

The appropriateness of this simple approach compared to more complex methods was demonstrated several years ago (Basketter et al, 1999). Since that time, others have examined similar approaches, albeit with a non-OECD protocol, and have demonstrated that the outcome is the same as linear interpolation (van Och et al, 2000; De Jong et al, 2002). EC3 values for a large number of chemicals have now been published, much being collated in the seminal paper from 2005 on 211 substances, which also shows that these values span several (about 5) orders of magnitude (Gerberick et al, 2005). Subsequent to this, further EC3 values have been published (Betts et al, 2005; Anderson et al, 2007; Basketter et al, 2007b; Dearman et al, 2007; SCCP 2007). To date, the lowest value (most potent allergen) is benz(a)pyrene with an EC3 of 0.0009% and the highest value among sensitizers is 89% for aniline. The dataset comprises 42 non-sensitizers; 66 weak sensitizers; 69 moderate sensitizers; 21 strong sensitizers; and 13 extreme sensitizers if one used the categorization scheme proposed by ECETOC (Kimber et al, 2003).

It has also been noted also that the 211 chemicals reported with EC3 values in the 2005 publication span the full range of reactive chemistry associated with skin sensitisation (Roberts et al, 2006; Aptula et al, 2007). These workers have concluded that sensitizers fall into some 6 main categories with a modest number of special cases, all of which are populated by the >200 chemicals for which EC3 values have been derived. However, it is of course important also that a quantitative measure such as the LLNA EC3 value is robust and reproducible, within a laboratory, between laboratories and over time. These aspects are reviewed in the following paragraphs.

In the original validation of the LLNA, five laboratories used the assay with a set of sensitizers and non-sensitizers, and even with the technical variations which inevitably arose

in the detail of test conduct, came up with essentially identical threshold predictions on all the substances evaluated (Kimber et al, 1995; Loveless et al, 1996). It should be noted that this work was done before the final definition of the OECD protocol and also before the final definition of how to derive the EC3 value in 1999. On this foundation, the reliability (robustness) of the prediction of EC3 values has been further assessed within single laboratories. Data have been published that reveal that the OECD positive control, hexyl cinnamic aldehyde (HCA), a weak sensitiser, gives reproducible EC3 values over time in an individual laboratory (Dearman et al, 2001). This has also been shown for other weak allergens (Basketter et al, 2007a). The reproducibility of EC3 values has also been tested at the opposite end of the potency spectrum, for the very strong allergen, p-phenylenediamine (PPD) which was assessed in each of two laboratories (Warbrick et al, 1999). EC3 values were highly consistent over each of 4 monthly determinations in each laboratory. Lastly, the EC3 value for a moderate allergen, isoeugenol, was assessed in a single laboratory (Basketter and Cadby, 2004).

The outcome of these various assessments supplemented with a small amount of additional unpublished data for 17 chemicals of widely varying skin sensitisation potency has been collated in Table 1. What is of particular note here is that, whilst there is of course biological variation in the EC3 determination (eg isoeugenol, where 31 determinations give a mean and standard error EC3 value of $1.5\% \pm 0.1\%$), the values typically lie well within their order of magnitude banding. Putting this differently, the variation in EC3 value for any given chemical tested in the same vehicle is substantially less than an order of magnitude, whereas when a wide range of skin sensitisers are examined, then EC3 values for substances of different potency span several orders of magnitude. Of course, vehicles can, and do, have an impact on derived EC3 values (reviewed in Basketter et al, 2001). However, the extent of this variation is usually no greater than the variation in EC3 values found with repeated measurements in the same vehicle (Table 1). A manuscript presenting a statistical evaluation confirming this is being finalised for submission to a suitable journal.

Table 1 Collation of EC3 data from repeat testing of 17 chemicals in multiple laboratories (data taken from Basketter et al, 2007a)

| Substance | EC3 values (%) | Vehicle ¹ | Mean EC3 (%) \pm SE ² |
|-----------------------------|---|----------------------|------------------------------------|
| Bandrowski's base | 0.04, 0.02 | AOO | 0.03 |
| 2,4-Dinitrochlorobenzene | 0.04, 0.02, 0.05, 0.03, 0.03, 0.02, 0.06, 0.03, 0.06, 0.05, 0.05, 0.06, 0.05 | AOO | 0.04 \pm 0.004 |
| Potassium dichromate | 0.05, 0.08, 0.14 | DMSO | 0.09 \pm 0.046 |
| p-Phenylenediamine | 0.07, 0.12, 0.09, 0.08, 0.06, 0.14, 0.06, 0.18, 0.16, 0.13 | AOO | 0.11 \pm 0.014 |
| 1,4-Hydroquinone | 0.11, 0.19, 0.12 | AOO | 0.14 \pm 0.04 |
| Methyldibromoglutaronitrile | 1.8, 0.9, 1.3 | AOO | 1.3 \pm 0.45 |
| Isoeugenol | 1.7, 1.1, 1.4, 1.3, 1.3, 1.0, 1.4, 1.5, 2.9, 0.8, 1.3, 1.6, 2.8, 0.9, 1.0, 1.7, 1.2, 1.4, 0.8, 2.1, 2.3, 1.1, 1.2, 1.2, 0.7, 1.0, 2.3, 1.3, 2.0, 1.6, 1.3 | AOO | 1.5 \pm 0.1 |
| Cinnamal | 3.1, 1.7, 2.7 | AOO | 2.3 \pm 0.4 |
| 1-Bromopentadecane | 5.2, 5.1 | AOO | 5.1 \pm 0.02 |
| L-Perillaldehyde | 8.1, 7.8 | AOO | 8.0 |
| Hexylcinnamal | 6.6, 11.3, 10.6, 4.4, 11.5, 8.8, 7.6, 11.0, 7.0, 10.6, 11.9, 11.7, 10.9, 11.7, 12.2 | AOO | 9.9 \pm 0.6 |
| Eugenol | 15.0, 4.9, 12.9, 7.5 | AOO | 10.1 \pm 2.3 |
| Abietic acid | 14.7, 8.3, 10.6 | AOO | 11.3 \pm 1.8 |
| Penicillin G | 16.7, 17.9, 30 | DMSO | 21.5 \pm 4.3 |
| Imidazolidinyl urea | 23.9, 31.2 | DMF | 27.6 |
| Hydroxycitronellal | 33.0, 27.5, 23.0 | AOO | 27.8 \pm 2.9 |
| 2-Ethylbutyraldehyde | 60, 76 | AOO | 68 |

¹AOO = acetone olive oil, 4:1, v/v; DMF = dimethyl formamide; DMSO = dimethylsulphoxide

²Numbers to no more than 2 significant figures; standard error not calculated if there were less than 3 data points.

Data to support that the LLNA EC3 is suitable for potency categorization and correlates with historical human data and clinical experience

The LLNA has been shown to be relevant as a model for the predictive identification chemicals with skin sensitization hazard. The protocol provides an objective measure of the crucial stage of the sensitisation process, the clonal expansion of lymphocytes that results from the application of a contact allergen by the appropriate route, epidermal application

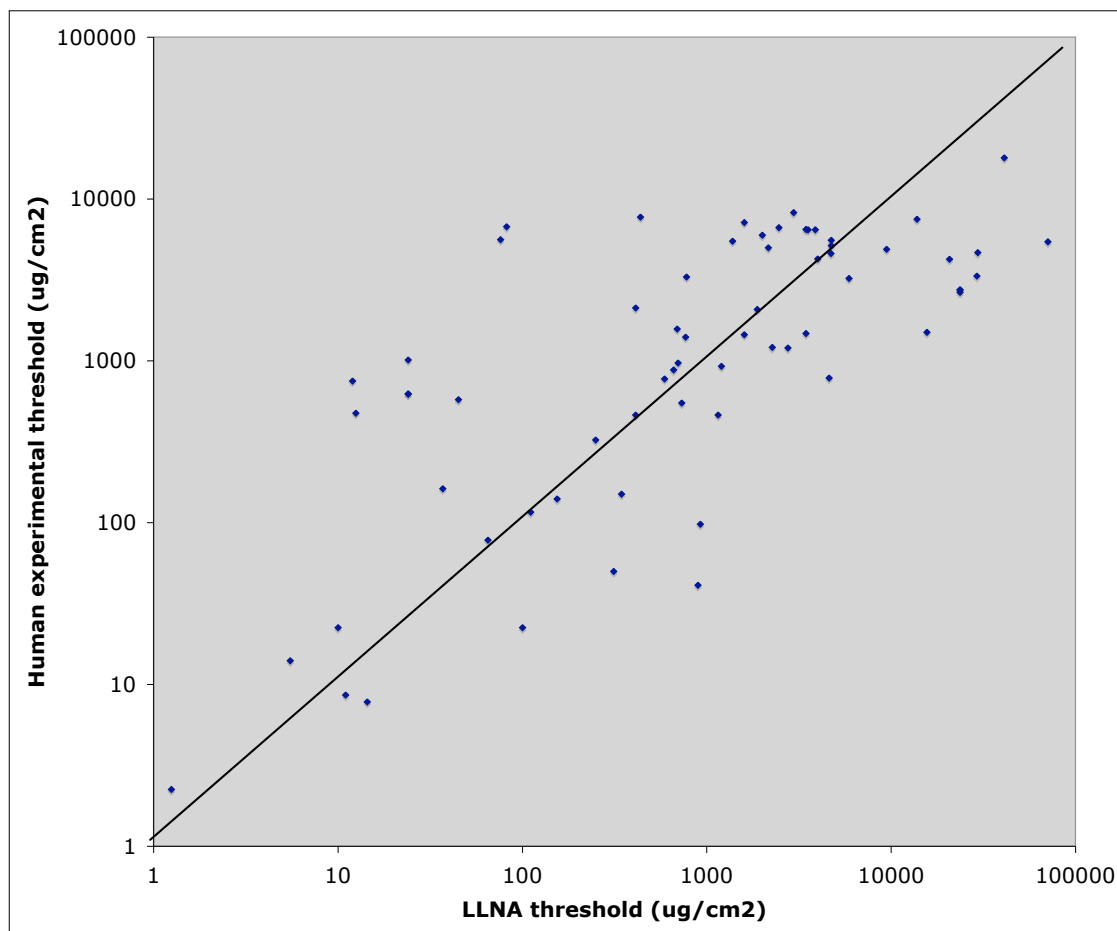
(Oort and Turk, 1965; Parrot and de Sousa, 1966). Both the route of administration and the immunological mechanisms involved are the same as those in man. The original validation of the LLNA contained a considerable number of known human sensitisers which were correctly identified by one or more of the contributing laboratories (NIH, 1999; Gerberick et al, 2000). This work was followed up with a specific study of a panel of known human contact allergens (n=11) which were correctly identified (Ryan et al, 2000). The quantitative element of the LLNA response was also noted some years ago (Kimber and Dearman, 1991). The method for the determination of the EC3 value having been fixed (see above), the relationship between LLNA EC3 values and human skin sensitisation potency was subsequently described.

Before reviewing this, two important points must be made: firstly, potency refers to the intrinsic property of a sensitising chemical, which is entirely independent from the frequency with which allergic contact dermatitis occurs in the general or a clinical population (since this depends heavily on exposure as well as potency); secondly, there is a paucity of data indicating the intrinsic potency of chemical skin sensitisers in humans, since this requires experimental studies of dubious ethics. Thus, the work that appears in the literature cannot offer the degree of certainty with regard to human/mouse correlations that would ideally be liked, and a degree of judgement is inevitable to help compensate for the relatively poor quality of the limited human data that are available. Hence, it has been important that many of the publications in this area have involved independent partners closely associated with the LLNA, including dermatologists, regulators and independent scientists (Hilton et al, 1998; Basketter et al, 1999, 2000, 2001, 2005; Gerberick et al, 2001; Griem, 2003; Schneider and Akkan, 2004).

The earlier potency comparisons referred to above tended only to assign human skin sensitisers into one of a number of categories (non, weak, moderate, strong, extreme) and to use the LLNA EC3 value to demonstrate that it was possible to assign the sensitising chemicals into these categories if certain cut-off limits were applied. Such an approach was strongly endorsed by industry groups (Kimber et al, 2001; 2003), by regulatory groups (Basketter et al, 2005) and most recently by the World Health Organisation (WHO, 2007). Although the outcome of this type of analysis could prove very useful, more interesting work was done by a number of groups who attempted to compare experimental thresholds in humans, typically a no effect level in a human repeated insult patch test (HRIPT) with the LLNA threshold, the EC3 value. Neither of these thresholds is of course absolute; they depend very much on the exposure conditions of the protocols. However, since each protocol is standardised, particularly the LLNA, then they represent a reasonable point of departure for such comparisons. Two groups have published such comparisons in 2003 and 2004. In one study, over 50 substances were assessed and a relationship between the LLNA and HRIPT thresholds shown (Schneider and Akkan, 2004). In a second study, a slightly different approach was chosen, but again a good relationship was demonstrated (Griem, 2003). Lastly, in a more recent analysis, a very critical approach was taken to selection of human data to try to ensure that only good quality HRIPT threshold information was used (Basketter et al, 2005). This restricted the analysis to just 25 substances, but again a good relationship between EC3 values and HRIPT thresholds was shown. In order to directly compare EC3 values, which are calculated as % concentration, to HRIPT thresholds, data from both test methods are expressed as dose per unit areas ($\mu\text{g}/\text{cm}^2$).

From these publications, it is possible to assemble all of the human intrinsic potency thresholds (ie the data from predictive human assays) and to compare them with LLNA EC3 values for the same chemicals. This is shown in figure 1.

Figure 1 Plot of human experimental thresholds v LLNA EC3 values



It is clear from this figure that there is a relationship between the two thresholds. The fact that the points fit well with the diagonal is also encouraging. It is our view that most of the variability in the dataset derives from the human studies. Within the publications reporting these data (Gerberick et al, 2001; Griem, 2003; Schneider and Akkan, 2004 and Basketter et al, 2005), several assumptions have had to be made. Furthermore, the human data were not produced to a well standardised protocol. Both of these factors are likely to contribute markedly to the spread of the human data.

It should be noted that the comparison of human and murine thresholds in Figure 1 comprises some 66 chemicals which cover a very wide spread of potency. The data underlying the figure is contained in Table 2. The threshold data for humans does not represent safe levels for occupational/consumer exposure.

Table 2 Human experimental v LLNA EC3 thresholds

| Substance | Human threshold ($\mu\text{g}/\text{cm}^2$) | Murine threshold ($\mu\text{g}/\text{cm}^2$) |
|------------------------------|---|--|
| MCI/MI | 1.25 ^a | 2.25 |
| Dinitrochlorobenzene | 5.5 ^b | 14 |
| Phenylenediamine | 10 ^c | 22.5 |
| Beryllium sulfate | 11 ^b | 8.6 |
| Phenylacetaldehyde | 12 ^{d,e} | 750 |
| Methylisothiazolinone | 12.5 ^a | 475 |
| Tetrachlorosalicylanilide | 14.4 ^b | 7.8 |
| Methyl-2-nonynoate | 24 ^a | 625 |
| Methyloctine carbonate | 24 ^a | 625 |
| trans-2-Hexenal | 24 ^a | 1012 |
| Formaldehyde | 37 ^a | 162.5 |
| Benzoisothiazolione | 45 ^a | 575 |
| Gold chloride | 65 ^b | 78 |
| Penicillin | 76 ^b | 5606 |
| Streptomycin | 82 ^b | 6750 |
| Glutaraldehyde | 100 ^a | 22.5 |
| Potassium dichromate | 111 ^b | 116 |
| Nickel sulfate | 154 ^c | 140 |
| Isoeugenol | 250 ^a | 325 |
| Cobalt sulfate | 313 ^b | 50 |
| Glyoxal | 345 ^b | 150 |
| Diethylenetriamine | 411 ^b | 463 |
| Methylanisylidene acetone | 412 ^b | 2123 |
| Butylglycidylether | 437 ^b | 7725 |
| Cinnamic aldehyde | 591 ^a | 775 |
| Thioglycerol | 661 ^b | 878 |
| Phenylpropionaldehyde | 692 ^b | 1575 |
| Oakmoss | 700 ^a | 970 |
| Ethylenediamine | 732 ^b | 550 |
| Dihydrocoumarin | 769 ^b | 1402 |
| Citral | 775 ^{a,f} | 3300 |
| Benzoyl peroxide | 895 ^b | 41 |
| Mercury bichloride | 924 ^b | 98 |
| Chlorpromazine | 1150 ^b | 463 |
| Benzylidene Acetone | 1200 ^c | 925 |
| Methylhydrocinnamal | 1379 ^a | 5500 |
| Diethylmaleate | 1600 ^c | 1450 |
| Ethyl acrylate | 1600 ^c | 7175 |
| Kanamycin | 1874 ^b | 2075 |
| Imidazolidnlyl urea | 2000 ^c | 5975 |
| Pentachlorophenol | 2155 ^b | 5000 |
| Mercaptobenzothiazole | 2269 ^b | 1214 |
| Aniline | 2463 ^{b,g} | 6658 |
| Farnesol | 2755 ^a | 1200 |
| Hydroxycitronellal | 2953 ^{a,h} | 8250 |
| Anisyl alcohol | 3448 ^a | 1475 |
| Methylhexanedione | 3448 ^c | 6500 |
| Acetyl isovaleryl | 3541 ^b | 6450 |
| Geraniol | 3875 ^{a,i} | 6475 |
| Lyril HMPCC | 4000 ^a | 4275 |
| Tetramethylthiuramdisulphide | 4610 ^b | 785 |
| Benzyl cinnamate | 4720 ^a | 4600 |
| Cinnamic alcohol | 4724 ^{c,j} | 5150 |

| Substance | Human threshold ($\mu\text{g}/\text{cm}^2$) | Murine threshold ($\mu\text{g}/\text{cm}^2$) |
|-----------------------------|---|--|
| Cyclamen aldehyde | 4724 ^a | 5575 |
| Eugenol | 5905 ^a | 3225 |
| Phenyl benzoate | 9448 ^a | 4900 |
| Linalool | 13793 ^a | 7500 |
| Neomycin sulfate | 15625 ^b | 1500 |
| Benzylbenzoate | 20690 ^a | 4250 |
| Alpha Amyl cinnamic alcohol | 23622 ^{d,k} | 2650 |
| Amylcinnamic aldehyde | 23622 ^a | 2750 |
| Hexylcinnamic aldehyde | 23622 ^a | 2750 |
| Benzocaine | 29167 ^b | 3338 |
| Lilial | 29525 ^{c,l} | 4675 |
| Pyridine | 41051 ^b | 17975 |
| isoMethylionone | 70866 ^a | 5450 |

Abbreviations: DSA₀₅ = dose per unit area of skin leading to a sensitization incidence of 5% calculated by linear interpolation from published HRIPT or HMT data; EC3 = estimated concentration of test substance necessary to produce an SI of 3; HMT = human maximization Test; HRIPT = Human repeated insult patch test; IFRA = International Fragrance Research Association; LLNA = murine local lymph node assay; LOEL = lowest observed effect level; NOEL = no observed effect level; SI = stimulation index.

^a Value is a NOEL.

^b Value is a DSA₀₅ (Schneider and Akkan, 2004)

^c Value is a LOEL.

^d Source for this datum could not be verified.

^e An HRIPT NOEL of 591 is reported in the IFRA std (see <http://www.ifraorg.org/index>).

^f An HRIPT NOEL of 1400 is reported in the IFRA std (see <http://www.ifraorg.org/index>).

^g A LOEL of 1379 is reported in Griem et al (2003).

^h An HRIPT NOEL of 5000 is reported in the IFRA std (see <http://www.ifraorg.org/index>).

ⁱ An HRIPT NOEL of 11,811 is reported in the IFRA std (see <http://www.ifraorg.org/index>).

^j An HRIPT NOEL of 3000 is reported in the IFRA std (see <http://www.ifraorg.org/index>).

^k An HRIPT NOEL of 3543 is reported in the IFRA std (see <http://www.ifraorg.org/index>).

^l An HRIPT NOEL of 4125 is reported in the IFRA std (see <http://www.ifraorg.org/index>).

Data to support the utility of the LLNA EC3 value (potency determination) in quantitative risk assessments for skin sensitization

For completeness, this section provides a succinct overview of how EC3 values might deliver value with respect to risk assessment and risk management. Two general possibilities have been considered. The first is placement of skin sensitising chemicals into one of a number of categories based on their potency (eg Gerberick et al, 2001; Kimber et al, 2003; Basketter et al, 2005). There are small differences between these various proposals, but all accept that skin sensitisers cover a very wide spectrum of relative potency and that strong and extreme allergens should be differentiated from moderate and weak allergens. It is known that the OECD is working on this concept and that the World Health Organisation convened an expert group which came to a similar, but as yet, unpublished, conclusion.

The second possibility is that the LLNA EC3 value can be used as a starting point for risk assessment (Kimber and Basketter, 1997). This option has been developed as fully as categorisation, but has the benefit of having also been implemented. The basic approach to the use of EC3 values in a quantitative risk assessment (QRA) has been outlined in a sequence of publications (Gerberick et al, 2001, Felter et al, 2002 and 2003). Use of the approach has been then detailed in several further publications (Basketter et al, 2003 and 2007; Zachariae, 2003; Corea et al, 2006; Api et al, 2007; Jowsey et al, 2007; www.ifraorg.org, 2007).

In principle, QRA for skin sensitisation follows the general principles of many toxicology endpoints: the determination of a no effect level in the animal model and then employment of a series of uncertainty factors to predict a safe exposure level for humans. The QRA approach as currently deployed identifies an acceptable daily exposure for specific skin sensitiser in a particular product use scenario. No doubt it could be modified to identify a general upper limit for daily exposure to a particular skin allergen, remembering always that this figure must be expressed in terms of dose per unit area. More detailed discussion of this topic can be found elsewhere (Kimber et al, 2007).

Authors' response to the questions

Q1: In those circumstances where an evaluation of skin sensitization potency is required for risk assessment purposes, do EC3 values derived from linear interpolation of LLNA dose response provide an appropriate and reliable approach?

A1: It is the view of the authors of this document that LLNA EC3 values do provide an appropriate and reliable approach.

Q2: If yes, do EC3 values provide a suitable method for ranking of contact allergens according to skin sensitisation potency?

A2: It is the view of the authors of this document that EC3 values do permit a useful ranking of contact allergens according to skin sensitisation potency. Given that EC3 values span some 5 orders of magnitude, it is further noted that ranking into a similar number of categories should be possible.

Q3: If yes, does ranking of potency based on LLNA-derived EC3 values correlate with available human data and clinical experience?

A3: It is the view of the authors of this document that relative potency in the mouse correlates well with human data, always bearing in mind that the latter are available only in limited quantities and are not always of good quality.

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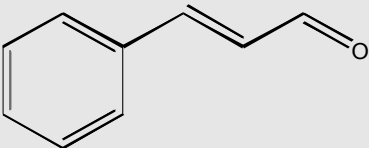
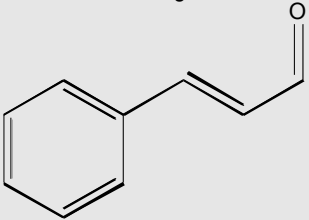
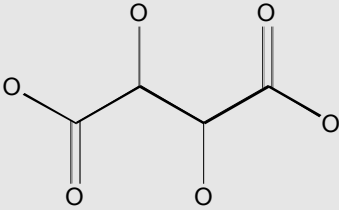
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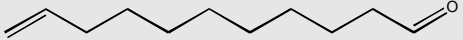
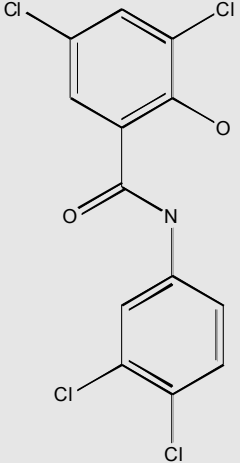
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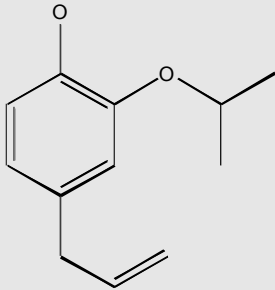
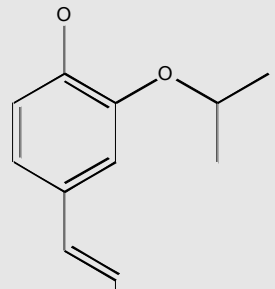
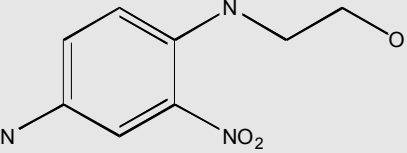
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Appendix 1 Tabulation of corrections to Gerberick et al, 2005 database plus 31 additional chemicals

Table of Corrections

| CHEMICAL STRUCTURE | CAS # | Vehicle ¹ | LLNA % | LLNA % | LLNA % | LLNA % | LLNA % | LLNA % | LLNA SI | LLNA SI | LLNA SI | LLNA SI | LLNA SI | LLNA SI | LLNA EC3 % | Potency category | Ref. |
|--|------------|----------------------|--------|--------|--------|--------|--------|--------|---------|---------|---------|---------|---------|---------|----------------|---|------|
| <div></div> <div>Cinnamic aldehyde</div> | 104-55-2 | AOO | 0.5 | 1.0 | 2.5 | 5.0 | 10.0 | | 1.4 | 0.9 | 1.9 | 7.1 | 15.8 | 3.0 | moderate | Basketter DA, Wright ZM, Warbrick EV, et al. Human potency predictions for aldehydes using the local lymph node assay. Contact Derm 2001; 45:89-94. | |
| <div>DELETE in original table</div> <div></div> <div>3-Phenyl propenal</div> | 14371-10-9 | AOO | 1.0 | 2.5 | 5.0 | 10.0 | 25.0 | | 2.4 | 4.7 | 8.8 | 10.2 | 13.1 | 1.4 | moderate | Patlewicz G, Wright ZM, Basketter DA, et al. Structure-activity relationships for selected fragrance allergens. Contact Derm 2002; 47:219-226. | |
| <div></div> <div>Tartaric acid</div> | 87-69-4 | DMF | 5 | 10 | 25 | | | | 1.0 | 0.9 | 1.5 | | | | non-sensitizer | UL unpublished | |

| | | | | | | | | | | | | | | | | | |
|---|-----------|---------|------|------|------|------|------|--|------|------|------|-----|-----|--|-------------------|----------|--|
|  <p>Undec-10-enal</p> | 112-45-8 | AOO | 5.0 | 10.0 | 25.0 | 50.0 | 75.0 | | 1.7 | 5.3 | 7.5 | 8.7 | 8.8 | | 6.8 | moderate | Patlewicz G, Wright ZM, Basketter DA, et al. Structure-activity relationships for selected fragrance allergens. Contact Derm 2002; 47:219-226. |
|  <p>3, 3', 4', 5-Tetrachlorosalicylanilide</p> | 1154-59-2 | Acetone | 0.25 | 0.5 | 1.0 | | | | 11.2 | 14.4 | 18.0 | | | | 0.04 ³ | extreme | Basketter DA, Scholes EW, and Kimber I. The performance of the local lymph node assay with chemicals identified as contact allergens in the human maximization test. Fd Chem Tox 1994; 32:543-547. |

| | | | | | | | | | | | | | | | | | |
|---|-------------|-----|------|------|------|-----|-----|--|-----|-----|------|-----|-----|--|------------------|----------------|--|
|  <p>Isopropyl eugenol</p> | 51474-90-9 | AOO | 12.0 | 29.0 | 59.0 | | | | 1.8 | 1.8 | 2.2 | | | | NC | non-sensitizer | Bertrand F, Basketter DA, Roberts DW, and Lepoittevin J-P. Skin sensitization to eugenol and isoeugenol in mice: possible metabolic pathways involving ortho-quinone and quinone methide intermediates. Chem Res Toxicol 1997; 10:335-343. |
|  <p>Isopropyl isoeugenol</p> | 186743-30-6 | AOO | 0.6 | 1.2 | 3.0 | | | | 3 | 5.7 | 10.7 | | | | 0.6 ³ | strong | Bertrand F, Basketter DA, Roberts DW, and Lepoittevin J-P. Skin sensitization to eugenol and isoeugenol in mice: possible metabolic pathways involving ortho-quinone and quinone methide intermediates. Chem Res Toxicol 1997; 10:335-343. |
|  <p>2-(4-Amino-2-nitro-phenylamino)-ethanol (HC Red No3)</p> | 2871-01-4 | AOO | 0.1 | 0.25 | 0.5 | 1.0 | 2.5 | | 0.5 | 1.2 | 1.9 | 1.8 | 3.3 | | 2.2 | moderate | Estrada E, Patlewicz G, Chamberlain M, et al. Computer-aided knowledge generation for understanding skin sensitization mechanisms: the TOPS-MODE approach. Chem Res Toxicol. 2003; 16:1226-1235 |

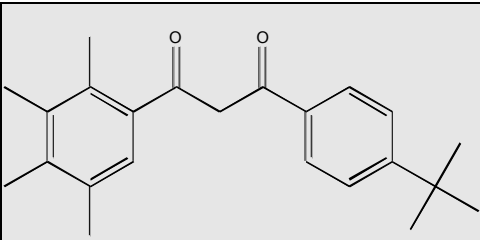
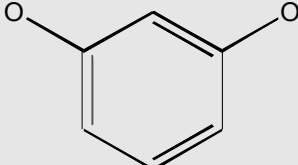
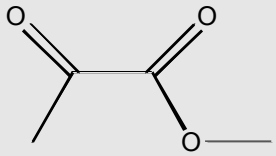
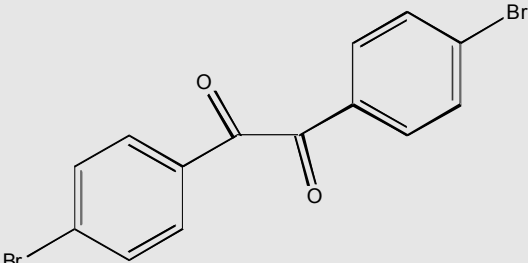
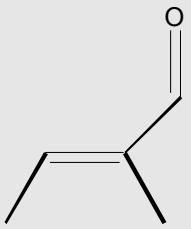
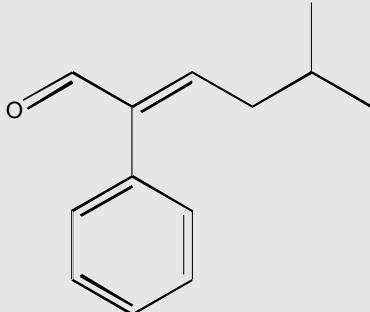
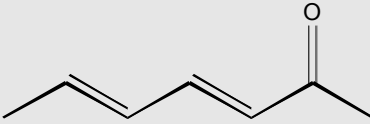
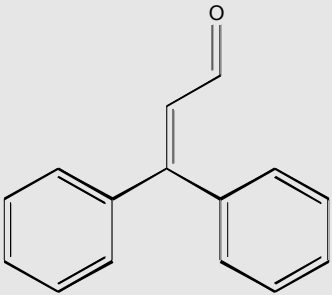
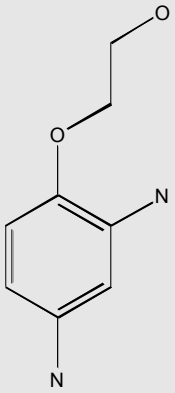
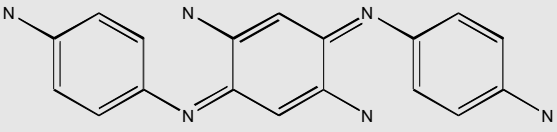
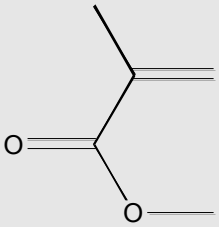
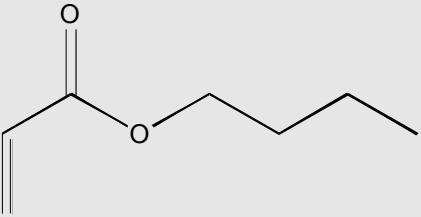
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|--|-----------|---------|------|------|------|------|------|------|-----|-----|-----|-----|------|------|-----|----------|---|-----------------|
|  <p>1-(2',3',4'5'-Tetramethylphenyl)-3-(4'-tertbutylphenyl) propane-1,3-dione</p> | Not known | Acetone | 10.0 | 20.0 | 40.0 | | | | | 1.6 | 1.2 | 1.6 | | | | NC | non-sensitizer | P&G unpublished |
|  <p>Resorcinol</p> | 108-46-3 | AOO | 1.0 | 2.5 | 5.0 | 10.0 | 25.0 | 50.0 | 1.8 | 2.3 | 2.6 | 6.3 | 10.1 | 12.5 | 5.5 | moderate | Basketter DA., Sanders D., Jowsey IR., Contact Dermatitis 2007: 56: 196-200 | |

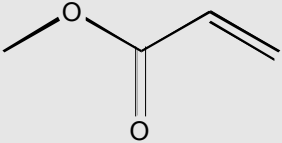
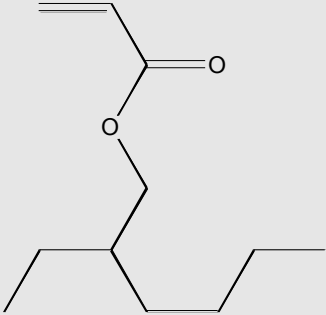
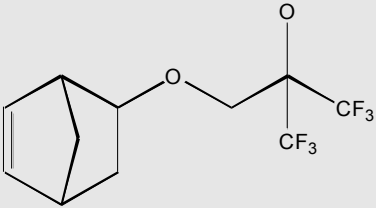
Table of new structures

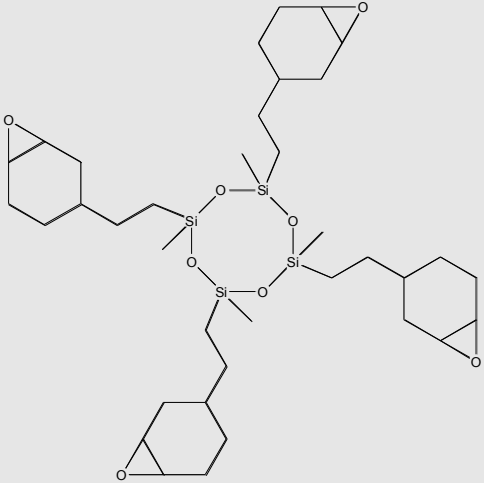
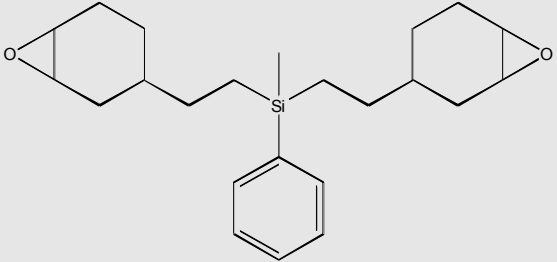
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|--|------------|----------------------|--------|--------|--------|--------|--------|--------|---------|---------|---------|---------|---------|---------|------------|------------------|----------------|
| <div></div> <div>Methyl pyruvate</div> | 600-22-6 | AOO | 1 | 2.5 | 5 | 10 | | | 1.2 | 3.1 | 4.7 | 8.0 | | | 2.4 | | UL unpublished |
| <div></div> <div>4,4-Dibromobenzil</div> | 35578-47-3 | AOO | 5 | 10 | 25 | 50 | | | 1.5 | 1.6 | 3.6 | 5.7 | | | 20.5 | | UL unpublished |

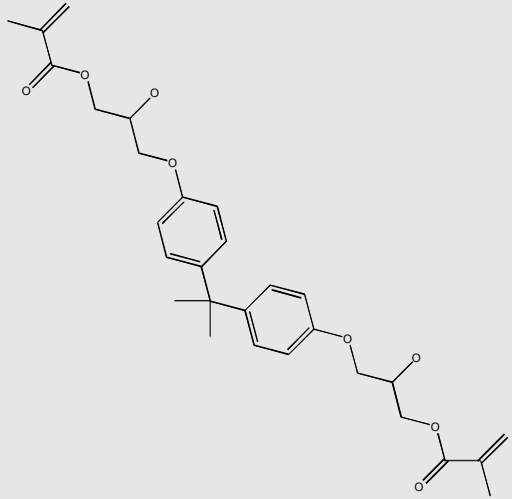
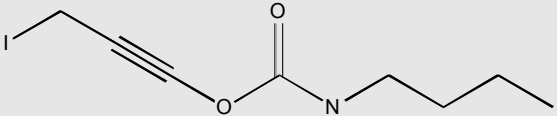
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|--|------------|-----|-----|----|-----|---|----|--|--|-----|-----|-----|-----|------|--|-----|--|----------------|
|  <p>Trans-2-methyl-2-butenal</p> | 497-03-0 | AOO | 10 | 25 | 50 | | | | | 1.5 | 1.0 | 2.8 | | | | ? | | UL unpublished |
|  <p>5-Methyl-2-phenyl-2-hexenal</p> | 21834-92-4 | AOO | 0.5 | 1 | 2.5 | 5 | 10 | | | 1.0 | 1.3 | 0.5 | 3.8 | 17.7 | | 4.4 | | UL unpublished |
|  <p>2,4-Hexadienal</p> | 142-83-6 | AOO | 0.5 | 1 | 2.5 | 5 | 10 | | | 0.9 | 1.5 | 2.2 | 4.2 | 14.8 | | 3.5 | | UL unpublished |

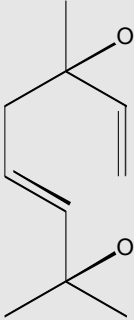
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|--|------------|-----|------|-------|------|-----|------|--|-----|-----|-----|-----|------|------|--|----------------|
|  <p>β-Phenylcinnamaldehyde</p> | 1210-39-5 | AOO | 0.1 | 0.25 | 0.5 | 1 | 2.5 | | 2.0 | 2.3 | 1.9 | 5.9 | 10.6 | 0.6 | | UL unpublished |
|  <p>2HCl</p> <p>2,4-Diaminophenoxyethanol HCl</p> | 66422-95-5 | AOO | 1 | 2.5 | 5 | 10 | 25 | | 1.6 | 1.6 | 2.7 | 5.7 | 8.3 | 5.5 | | UL unpublished |
|  <p>Bandrowski's base</p> | 20048-27-5 | AOO | 0.01 | 0.025 | 0.05 | 0.1 | 0.25 | | 1.1 | 3.1 | 5.7 | 6.5 | 5.6 | 0.04 | | UL unpublished |

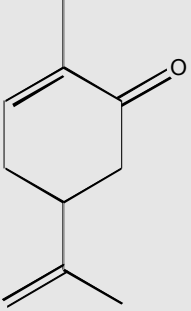
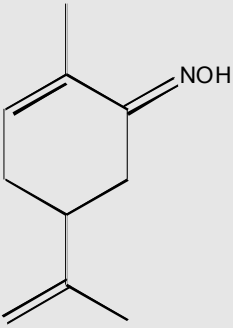
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|---|----------|-----|----|-----|----|----|-----|--|-----|-----|-----|-----|-----|--|----|------|---|
|  <p>methylmethacrylate</p> | 80-62-6 | AOO | 10 | 30 | 50 | 75 | 100 | | 1.4 | 1.5 | 1.5 | 2.1 | 3.6 | | 90 | weak | <p>Betts CJ, Dearman RJ, Heylings JR, Kimber I and Basketter DA. Skin sensitization potency of methyl methacrylate in the local lymph node assay : comparisons with guinea pig data and human experience. Contact Derm 2006; 55: 140-127.</p> |
|  <p>Butyl acrylate</p> | 141-32-2 | AOO | 1 | 2.5 | 5 | 10 | 25 | | 0.7 | 1.3 | 1.4 | 2.5 | 8.7 | | 11 | weak | <p>Dearman RJ et al. Comparative analysis of skin sensitisation potency of acrylates (methyl acrylate, ethyl acrylate, butyl acrylate and ethylhexyl acrylate) using the local lymph node assay. Submitted for publication</p> |

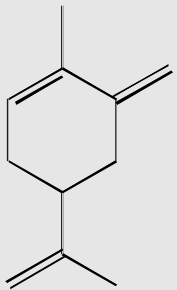
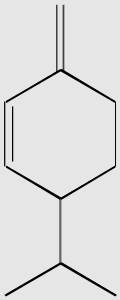
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|--|-------------|-----|-----|-----|-----|----|-----|--|-----|-----|-----|-----|-----|--|----|------|---|
|  <p>Methyl acrylate</p> | 96-33-3 | AOO | 1 | 2.5 | 5 | 10 | 25 | | 0.8 | 0.8 | 1.3 | 1.6 | 3.8 | | 20 | weak | Dearman RJ et al. Comparative analysis of skin sensitisation potency of acrylates (methyl acrylate, ethyl acrylate, butyl acrylate and ethylhexyl acrylate) using the local lymph node assay. Submitted for publication |
|  <p>Ethylhexyl acrylate</p> | 103-11-7 | AOO | 0.5 | 1 | 2.5 | 5 | 10 | | 1.1 | 1.2 | 0.9 | 1.2 | 3.1 | | 10 | weak | Dearman RJ et al. Comparative analysis of skin sensitisation potency of acrylates (methyl acrylate, ethyl acrylate, butyl acrylate and ethylhexyl acrylate) using the local lymph node assay. Submitted for publication |
|  <p>2-[(bicyclo[2.2.1]hept-5-ene-2-yloxy)methyl]-1,1,1,3,3,3-hexafluoro-2-propanol (norbornene fluoroalcohol)</p> | 305815-63-8 | AOO | 5 | 10 | 25 | 50 | 100 | | 0.7 | 0.8 | 1.9 | 3.2 | 3.7 | | | | DeLorme MP, Ladics GS, Donner EM, Wagner VO, Finlay C, Frame SR, Everds NE, Loveless SE. Acute, subchronic and mutagenicity studies with norbornene fluoroalcohol. Drug Chem Toxicol 2005; 28: 379-395 |

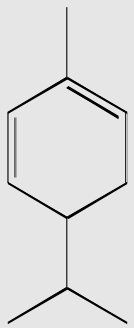
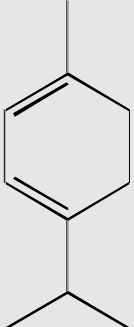
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|  <p>3,4-epoxycyclohexylethyl-cyclopolydimethylsiloxane (Tet-sil)</p> | | AOO | 50 | 100 | | | | | 1.2 | 1.2 | | | | | | <p>Kostoryz EL, Zhu Q, Zhao H, Miller M and Eick JD. Assessment of the relative skin sensitization potency of siloranes and bis-GMA using the local lymph node assay and QSAR predicted potency. J Biomed Mat Res A 2006; 79: 684-688</p> |
|  <p>Bis-3,4-epoxycyclohexylethyl-phenyl-dimethylsilane (Ph-Sil)</p> | | AOO | 25 | 35 | 50 | | | | 3.7 | 4.2 | 7.9 | | | | | <p>Kostoryz EL, Zhu Q, Zhao H, Miller M and Eick JD. Assessment of the relative skin sensitization potency of siloranes and bis-GMA using the local lymph node assay and QSAR predicted potency. J Biomed Mat Res A 2006; 79: 684-688</p> |

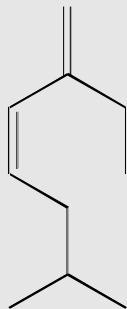
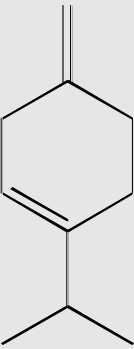
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|  <p>2,2-bis[4-(2-hydroxy-3 methacryloxypropoxy)phenyl]-propane (Bis-GMA)</p> | 1565-94-2 | AOO | 35 | 75 | | | | | 2.0 | 5.9 | | | | | | <p>Kostoryz EL, Zhu Q, Zhao H, Miller M and Eick JD. Assessment of the relative skin sensitization potency of siloranes and bis-GMA using the local lymph node assay and QSAR predicted potency. J Biomed Mat Res A 2006; 79: 684-688</p> |
|  <p>Iodopropynyl butylcarbamate</p> | 87977-28-4 | AOO | 0.1 | 1 | 5 | 10 | | 0.7 | 3.4 | 4.2 | 12 | | | | | <p>Siebert J. The sensitizing potential of iodopropynyl butylcarbamate in the local lymph node assay. Contact Derm 2004; 51: 318-319</p> |

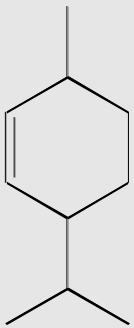
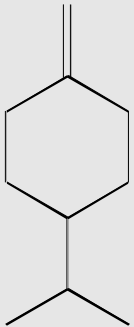
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| <p>Linalool aldehyde</p> | | AOO | 1 | 5 | 15 | | | | 1.2 | 2.0 | 4.2 | | | | | <p>Sköld M., Börje A., Harambasic E., Karlberg A.-T., Contact Allergens Formed on Air Exposure of Linalool. Identification and Quantification of Primary and Secondary Oxidation Products and the Effect on Skin Sensitization. Chem. Res. Toxicol. 2004, 17, 1697-1705</p> |
| <div data-bbox="436 792 569 1109">  </div> <p>Linalool alcohol</p> | | AOO | 1 | 10 | 30 | | | | 1.0 | 1.3 | 1.3 | | | | | <p>Sköld M., Börje A., Harambasic E., Karlberg A.-T., Contact Allergens Formed on Air Exposure of Linalool. Identification and Quantification of Primary and Secondary Oxidation Products and the Effect on Skin Sensitization. Chem. Res. Toxicol. 2004, 17, 1697-1705</p> |

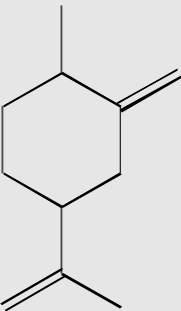
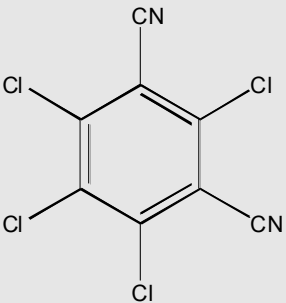
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|---|--|-----|-----|----|----|--|--|--|-----|-----|-----|--|--|--|--|--|
|  <p>R-Carvone</p> | | AOO | 6 | 12 | 20 | | | | 1.3 | 2.6 | 6.2 | | | | | <p>Nilsson A.-M., Andresen Bergström M., Luthman K., Nilsson J.L.G., Karlberg A.-T. An a,b-unsaturated oxime identified as a strong contact allergen. Indications of antigen formation via several pathways. Food and Chem. Toxicol. 43 (2005) 1627-1636</p> |
|  <p>R-Carvoxime</p> | | AOO | 0.1 | 1 | 5 | | | | 2.1 | 3.7 | 8.1 | | | | | <p>Nilsson A.-M., Andresen Bergström M., Luthman K., Nilsson J.L.G., Karlberg A.-T. An a,b-unsaturated oxime identified as a strong contact allergen. Indications of antigen formation via several pathways. Food and Chem. Toxicol. 43 (2005) 1627-1636</p> |

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|--|--|--|-----|-----|----|----|--|--|--|--|------|-----|-----|--|--|--|--|--|--|
|  <p>(5R)-5-Isopropenyl-2-methyl-1-methylene-2-cyclohexene</p> | | | AOO | 0.5 | 5 | 15 | | | | | 0.94 | 1.9 | 6.6 | | | | | | <p>Nilsson A.-M., Andresen Bergström M., Luthman K., Nilsson J.L.G., Karlberg A.-T. A Conjugated Diene Identified as a Prohaptens: Contact Allergenic Activity and Chemical Reactivity of Proposed Epoxide Metabolites. Chem. Res. Toxicol. 2005, 18, 308-316.</p> |
|  <p>b- Phellandrene</p> | | | AOO | 1 | 10 | 20 | | | | | 1.1 | 4.8 | 23 | | | | | | <p>Andresen Bergström M., Luthman K., Nilsson J.L.G., Karlberg A.-T. Conjugated Dienes as prohaptens in Contact Allergy: In Vivo and in Vitro Studies of Structure- Activity Relationships, Sensitizing Capacity, and Metabolic Activation. Chem. Res. Toxicol. 2006, 19, 760-769.</p> |

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|--|--|-----|---|----|----|----|----|--|-----|-----|-----|-----|----|--|--|---|
|  <p data-bbox="428 621 577 643">α- Phellandrene</p> | | AOO | 1 | 10 | 25 | | | | 1.1 | 5 | 28 | | | | | <p data-bbox="1753 196 1900 699">Andresen Bergström M., Luthman K., Nilsson J.L.G., Karlberg A.-T. Conjugated Dienes as prohaptens in Contact Allergy: In Vivo and in Vitro Studies of Structure-Activity Relationships, Sensitizing Capacity, and Metabolic Activation. Chem. Res. Toxicol. 2006, 19, 760-769</p> |
|  <p data-bbox="428 1143 577 1164">α- Terpinene</p> | | AOO | 1 | 5 | 10 | 15 | 25 | | 1.1 | 1.5 | 3.4 | 8.9 | 23 | | | <p data-bbox="1753 709 1900 1213">Andresen Bergström M., Luthman K., Nilsson J.L.G., Karlberg A.-T. Conjugated Dienes as prohaptens in Contact Allergy: In Vivo and in Vitro Studies of Structure-Activity Relationships, Sensitizing Capacity, and Metabolic Activation. Chem. Res. Toxicol. 2006, 19, 760-769</p> |

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|---|--|-----|---|----|----|----|----|--|-----|------|------|------|-----|--|--|---|
|  <p>(4Z)-2-Methyl-6-methylenooct-4-ene</p> | | AOO | 1 | 5 | 10 | 15 | 25 | | 1.1 | 0.87 | 0.78 | 0.89 | 2.1 | | | Andresen Bergström M., Luthman K., Nilsson J.L.G., Karlberg A.-T. Conjugated Dienes as prohaptens in Contact Allergy: In Vivo and in Vitro Studies of Structure-Activity Relationships, Sensitizing Capacity, and Metabolic Activation. Chem. Res. Toxicol. 2006, 19, 760-769 |
|  <p>b- Terpinene</p> | | AOO | 1 | 10 | 25 | | | | 1.4 | 1.3 | 2.1 | | | | | Andresen Bergström M., Luthman K., Nilsson J.L.G., Karlberg A.-T. Conjugated Dienes as prohaptens in Contact Allergy: In Vivo and in Vitro Studies of Structure-Activity Relationships, Sensitizing Capacity, and Metabolic Activation. Chem. Res. Toxicol. 2006, 19, 760-769 |

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|--|--|-----|---|----|----|--|--|--|------|------|-----|--|--|--|--|--|---|
|  <p>(3S,6R)-3-isopropyl-6-methylcyclohexene</p> | | AOO | 1 | 10 | 25 | | | | 0.84 | 1.0 | 2.9 | | | | | | Andresen Bergström M., Luthman K., Nilsson J.L.G., Karlberg A.-T. Conjugated Dienes as prohaptens in Contact Allergy: In Vivo and in Vitro Studies of Structure-Activity Relationships, Sensitizing Capacity, and Metabolic Activation. Chem. Res. Toxicol. 2006, 19, 760-769 |
|  <p>4-Isopropyl-1-methylenecyclohexane</p> | | AOO | 1 | 10 | 25 | | | | 1.2 | 0.71 | 1.4 | | | | | | Andresen Bergström M., Luthman K., Nilsson J.L.G., Karlberg A.-T. Conjugated Dienes as prohaptens in Contact Allergy: In Vivo and in Vitro Studies of Structure-Activity Relationships, Sensitizing Capacity, and Metabolic Activation. Chem. Res. Toxicol. 2006, 19, 760-769 |

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|---|-----------|-----|-------|------|------|-----|-----|----|-----|-----|------|------|------|-----|--|--|---|
| <div></div> <div>(1R,4R)-4-Isopropenyl-1-methyl-2-methylenecyclohexane</div> | | | AOO | 1 | 5 | 10 | 15 | 25 | | 1.3 | 1.8 | 1.2 | 2.3 | 2.9 | | | Andresen Bergström M., Luthman K., Nilsson J.L.G., Karlberg A.-T. Conjugated Dienes as prohaptens in Contact Allergy: In Vivo and in Vitro Studies of Structure-Activity Relationships, Sensitizing Capacity, and Metabolic Activation. Chem. Res. Toxicol. 2006, 19, 760-769 |
| <div></div> <div>Chlorothalonil</div> | 1897-45-6 | DMF | 0.003 | 0.01 | 0.03 | 0.1 | 0.3 | | 2.1 | 9.4 | 13.8 | 18.4 | 27.2 | | | | Boman A., Montelius J., Rissanen R.-L., Liden C. Sensitizing potential of chlorothalonil in the guinea pig and the mouse. Contact Dermatitis, 2000, 43, 273-279. |